ANALYSIS OF EARLY PHASE WOUND HEALING AFTER ER: YAG LASER ASSISTED PERIODONTAL POCKET DEBRIDEMENT

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Abstract: The main effect of pocket debridement in periodontology is a tissue wounding followed by phases of healing. Complex chemically mediated amplification cascade is responsible for the beginning and control of the inflammatory response that can be activated by numerous events. The chemical mediators that have active contribution to this process are numerous. Main aim of this study was to make comparative histomorphometrical analysis of healing after soft tissue wall debridement on the periodontal pocket with (1) an erbium laser using low energy levels and with (2) conventional manual instrumentation in order to examine marker proteins during early stage of wound healing. Total number of 15 subjects with diagnosed chronic periodontitis and loss of attachment bigger than 5 mm on one side of the tooth were included in the study. Every patient was primarily treated with supra gingival removal of the dental plaque and calculus using an ultrasonic device and removal of all local irritating factors (if it's necessary). That was done in order to create an adequate condition for improving the oral hygiene. This was followed by subgingival "non-surgical debridement" using conventional- mechanical versus laser assisted treatment. During laser assisted treatment were used following energy parameters: for soft tissue, energy density is about 178 mJ / mm^2 and 256 mJ /mm^2 . The biopsy was taken from the soft tissue wall of the periodontal pocket, 24 and 72 hours after periodontal treatment and these marker proteins were assessed: myeloperoxidase, vimentin, CD34 and CD68. The results from this study showed that subjects treated with conventional method using hand instruments compared with the group of patients treated with laser after 24 hours have significant differences ($p < 0.001$) for all marker proteins. Also, similar results were obtained in the bioptical materials taken after 72 hours. Only the results for CD34 showed a significant difference ($p < 0.001$) for the laser assisted treatment group compared to the results from conventional manual instrumentation. Early stage of wound healing after low energy erbium laser assisted periodontal treatment was characterized by relatively reduced marker proteins in context of early phase wound healing for myeloperoxidase, vimentin and CD68, but with a higher expressed value for CD34. Most likely, these values for CD34 are related to minimally invasive periodontal pocket instrumentation and to the very narrow zone of soft tissue thermal damage. These results can be interpreted as a positive sign for the direction of the appropriate cell types to the inflammation area in a timely manner. But at the end, it must be noted that numerous facts are still needed to further clarify the biological mechanism of healing after the application of the low energy Er: YAG laser irradiation before the understanding of this complex is completed.
Keywords: Low-energy ER: YAG laser, early phase wound healing, laser assisted pocket debridement, marker proteins.

1. INTRODUCTION

Periodontitis is an inflammatory disease manifested by loss of connective tissue and bone support. This inflammatory process is characterized by exacerbations and remissions. Today is generally known that the pathogenic bacteria from the dental biofilm and the susceptible host produce a complex inflammatory / immune response that results in a clinical expression of the periodontal inflammation. This process cause catabolic changes of integral tissue entities that lead to formation of periodontal pocket, as main clinical sign of the periodontitis. (Newman et al, 2002) Therapeutic strategies for the effective periodontal treatment are primarily targeted toward removal or reduction of the inflammatory process of the disease by removing supra and subgingival dental plaque and establishing a local environment and microflora compatible with periodontal health. Clinical parameters used to determine if treatment is successful are: decreasing the periodontal pocket depth, maintaining or improving clinical attachment levels and reducing bleeding during probing. There are two therapeutic options for achieving the aforementioned, nonsurgical-conservative and surgical approaches in periodontal treatment.

Basically, nonsurgical periodontal therapy is most common in everyday dental practice and can be perform mechanically using conventional hand instruments. But, nowadays with introduction of the laser in dentistry, the laser-assisted periodontal therapy becomes an alternative or adjunctive therapy. Lasers have numerous physical properties affecting a broad range of biological responses and therefor are adequate for treating of the periodontal

diseases. All high power dental lasers can cause thermal side-effects. Most non-sporulating bacteria, such as the most of the periodontopathic anaerobes, are deactivated on 50°C. (Russell, 2003) At a temperature of 60°C the laser energy causes coagulation and hemostasis. Usage of lasers as a supplement to scaling and root planning may improve the effectiveness of non-surgical periodontal treatment. (AAP, 2002; Coluzzi, 2004) Also in the contemporary literature it is suggested that the erbium laser family present big range of application in everyday practice and are the most suitable lasers for periodontal therapy. (Yamaguchi et al, 1997; Cobb, 2006; Moghare Abed et al, 2007)

Through the past, numerous methods were designed to preserve epithelial migration on instrumented root surface, and the most common used methods are: soft-tissue curettage, various types of flap procedures including modified Widman flap, guided tissue regeneration and more recently lasers assisted debridement. The main aim of subgingival curettage is to remove the epithelium which is necessary for reattachment of periodontal tissue. (Maskow, 1966) Regardless which method of pocket debridement is used, the result is a tissue wounding followed by the process of healing. The most wanted outcome is reestablishment of normal homeostasis or complete resolution of the tissue. The physiologic resolution of the tissue is essential to maintain homeostasis at the cellular and tissue level. Inflammation is a normal and prerequisite to healing process.(Hardy, 1989) During acute phase of inflammation, a cascade of events at the molecular signaling level leading to the resolution phase, including cell migration, activation, proliferation, differentiation and clearance.(Serhan & Savil,2005; Gronert,2008) The complex chemically mediated amplification cascade is responsible for the start and control of the inflammatory response and can be activated by numerous events in the tissue. The inflammatory reactions can cause vascular and cellular cascades, occurring parallel and they are significantly interlinked. There are numerous chemical mediators that make an active contribution to this process. Most of the inflammatory processes are self-limiting and self-resolving, (Serhan et al,2007) suggesting the existence of endogenous anti-inflammatory and/or pro resolution mediators during the inflammation.(Serhan et al,2008) Acute inflammation is also characterized by the rapid influx of blood granulocytes, dominantly neutrophils and after short time followed by monocytes. Monocytes mature into inflammatory macrophages, proliferate and affect the functions of resident tissue macrophages. Resolution of inflammation can occur in two ways, if granulocytes are eliminated and the tissue mononuclear cells as macrophages and lymphocytes returns to normal number and phenotypes as before the inflammation. (Glorry, 2004)

The conversion of laser energy into heat and its scattering in the surrounding tissue leads to an increase in at least 45–50° C temperature gradient. This process generates supra physiologic level of heat, that is able to induce thermally temporary changes in cellular metabolism. These changes are characterized by the production of a small family of proteins named as heat shock proteins.(Souil et al, 2001) Considering all the facts and information from the contemporary dental medicine, the future concern of wound healing will depend on the temporal relations involved in the resolution of local acute inflammation and tissue injury at the cellular and molecular levels. Recent research suggests the presence of photo bioactive reactions when working with high-output laser at a low-power setting and a shortened exposure time. This so called Low-level laser treatment (LLLT) is frequently used today to accelerate healing.(Ohshiro& Calderhead, 1991) The photobioactive reaction can activate the proliferation and differentiation of cells and can occur as a result of using a high-output laser at a low-power setting. (Ohshiro& Calderhead, 1991) With this non-destructive thermal and non-thermal bio activation which occur at the periphery of the target tissue some of the advantages of laser light usage can be explain. (Aoki et al, 2008) The concept of laser assisted therapeutic approach, using low-energy level Er:YAG irradiation might be expected to facilitate or promote the normal tissue repair, and thereby enhance the sequence of events that take the tissues from their injured to their 'normal' state.

Main aim of this study was to make comparative histomorphometrical analysis of healing after soft tissue wall debridement on the periodontal pocket with (1) an erbium laser using low energy levels and with the (2) conventional manual instrumentation in order to examine marker proteins during early stage of wound healing.

2. MATERIAL AND METHOD

After a comprehensive periodontal examination 15 patients older than 35 years with generalized chronic periodontitis were selected as examined subjects. Inclusion criteria were: nonsmokers, no periodontal treatment within the last 6 months, no systemic disease influencing the outcome of therapy and no use of antibiotics prior to treatment. Split-mouth design was performed on all subjects and total number of 15 contralateral single and multi-rooted tooth were examined. Each tooth from the contra lateral pair must have attachment lost of at least 5 mm on one aspect of the tooth.

The active treatment consists of initial therapy- supragingival cleaning using ultrasonic device and creation of condition that enhance oral hygiene (if needed), followed by phase 1 of periodontal therapy-"non-surgical debridement". In the control group this non-surgical debridement was done by scaling (with ultrasonic device) and

root planning (with Gracey curettes) in addition with gingival curettage (removal of the pocket epithelium and infiltrated sub epithelial connective tissue), using complete set of Gracey curettes (Hu-Friedy Co.). In the tested group the non-surgical debridement was laser assisted 'scaling and root planning' performed with LiteTouch Er:YAG laser, manufactured by Syneron (Yokneam-illit, Israel). This laser had direct delivery system with active medium built into the hand piece base. On the root surface the laser was positioned with an inclination of the fiber tip of 10-15º to the vertical axis of the tooth. Instrumentation was preformed from the apical to coronal direction in parallel paths. Laser settings were: hard tissue, non-contact, laser energy 100mJ, pulse frequency 15 Hz, chisel tip x 17mm; water spry level 6; energy density about 256 mJ/ mm^2, power density about 3.85 watt/mm^2; pulse width about 170 microsec. The instrumentation for both hand instruments and laser was performed until the operator felt that the root surfaces were adequately planed and smooth.

The laser assisted periodontal pocket debridement also known as "gingival curettage" was performed on the soft tissues with the laser kept at 20-40º angle between the laser tip and the vertical axis of the tissue and with parallel movements along the pocket wall, starting from the bottom of the pocket. Laser settings were: soft tissue, non-contact mode, laser energy 50 mJ , pulse frequency 30 Hz, tip 0,6 x 17 mm; water spray 6; energy density about 178 mJ/mm^2; power density about 5.35 watt/mm^2; pulse width about 290 microsec. In this study for pocket debridement we used "light dose" in a defined laser periodontal protocol. (Gregg & McCarthy, 2001) Because of the fact that the Er:YAG laser haemostatic properties are considered as marginal (Kesler et al,2000) after completion of the laser assisted periodontal pocket debridement a slight pressure was applied until bleeding was stopped. (Nabers, 1966)

Following biomarkers were monitored:

(1) **Myeloperoxidase** (MPO) is a white blood cell-produced inflammatory enzyme that measures disease activity from the luminal aspect of the arterial wall. This enzyme is localized in azurophilic granules of polymorphonuclear neutrophils and macrophages, and is exported into extracellular fluid in every inflammatory process.(Loria et al, 2008) Every time, when the artery wall is damaged or inflamed, MPO is released by macrophages where it accumulates. MPO has been demonstrated to be a local mediator of tissue damage. (Ren & Zhang, 2005) Any uncontrolled degranulation highlights the inflammation and can also lead to tissue damage even in absence of inflammation. Several types of tissue injuries and the pathogenesis of several other major chronic diseases are linked with MPO-derived oxidants.(Khan et al, 2018) Thus, the level of MPO activity is one of the best diagnostic tools of inflammatory and oxidative stress among different chronic diseases. (Eriksson et al, 2009) Also, these findings have implicated that MPO is an important therapeutic target in the treatment of inflammatory conditions. In contrast to its injurious effects at sites of inflammation, recent studies of animal models of different inflammatory diseases demonstrated that MPO deficiency results in the excessive inflammatory response and that affects on neutrophil functions including cytokine production. (Aratani, 2018)

(2) **Vimentin** is a marker of mesenchymal-derived cells or cells undergoing an epithelial-to-mesenchymal transition. (Eeriksson et al, 2009) It also is expressed on the cell surface of apoptotic neutrophils (Moisan & Girard, 2006) According to this, vementin participate in the recognition process of apoptotic neutrophils by phagocytes for the resolution of inflammation and in the establishment of an acute inflammatory response *in vivo*. (Moisan et al, 2007)

(3) **CD34** cells has important role in the wound healing, due improving the wound healing, by decreasing the inflammatory reaction and increasing the neovascularization of the wound.(Pedroso et al,2011) The CD34 protein is member of a family of single-pass trans-membrane sialomucin proteins.(Nielsen & McNagny,2008) This protein was first described on hematopoietic stem as a cell surface glycoprotein and it main function was as a cell-cell adhesion factor. The cell surface protein CD34 is frequently used as a marker for positive selection of engrafting human hematopoietic stem, progenitor and endothelial cells.(Dao & Nolta,1999)

(4) **CD68** is a surface marker of macrophages.(Ren & Zhang, 2005) This enzyme plays a main role in the regulation of wound healing, inflammation and fibrosis. (Brochhausen et al, 2017)

Tissue biopsy was taken from the soft tissue wall of the periodontal pocket, 24 and 72 hours after performed periodontal treatment and myeloperoxidase, vimentin, CD34 and CD68 values were examined. The tissue specimens were formalin-fixed and paraffin embedded at Institute of Pathology, at the Medical faculty, Skopje. We used a routine hematoxylin-eosin stain and were performed additional immunohistochemically analysis, using the following antibodies, vendors and dilutions: myeloperoxidase (DAKO,1:300), Vimentin (DAKO,1:100), CD34 (DAKO,1:50) and CD68 (DAKO,1:100). After deparafinisation, and PTLink pretreatment (pH 6 or 9) for 60 min, sections were washed with PBS for 5min, followed by application of peroxidase blocking reagent for 5min. After washing in buffer for 5min, incubation by primary antibody for 20min was done. After double washing steps with PBS, sections were transferred to the secondary antibody for 20min. The sections were washed with PBS and incubated for 5min with diaminobenzidine. Slides were double rinsed and counter stained with hematoxylin, followed by dehydration in graded alcohol solutions. To standardize the results, positive cells and number of blood

vessels were counted in 3 fields (x400 magnification) using light microscope Olympus BX41. The selected fields were with highest density "hot spots" and the mean number was calculated.
The difference between the two groups was analyzed using T-test for dependent and non-dependent samples and Mann-Whitney U test (independent samples with irregular distribution).

3. RESULTS

The effects of low-level Er:YAG laser irradiation on soft tissue wall of periodontal pocket was evaluated to determine the acute phase of healing. The immunohistochemically analysis of tested samples demonstrates significant differences($p < .05$) in all parameters for hand instruments treated group after 24h vs.72h (Tab.No. 1). For laser treated group in Tab. No.2 may notice significant differences on all tested parameters ($p < .05$) after 24h vs. 72h.

	Mean	Std.Dv.	N	Diff.	Std.Dv.	T	df	p
Myelo/Cont/24.	108.1333	10.48037	15	-32.800	16.60120	-7.6521	14	0.000002
Myelo/Cont/72	140.9333	13.55658						
Vimen/Cont/24	76.9333	10.76016	15	-177.000	64.80520	-10.5781	14	0.000000
Vimen/Cont/72	253.9333	62.71417						
CD34Cont/24	13.5333	2.35635	15	-10.933	6.63827	-6.3789	14	0.000017
CD34/Cont/72	24.4667	5.96258						
CD68Cont/24	29.6000	4.22239	15	-94.267	19.93728	-18.3121	14	0.000000
CD68/Cont/72	123.8667	17.27453						

Tab. No. 1. Hand instrument treated group after 24 vs.72h

	Mean Contr.	Mean Examin.	t-value	df	p	Std.Dev. Contr.	Std.Dev. Examin.	F-ratio	p
Myelo/24.	108.1333	56.62500	10.84048	21	0.000000	10.48037	11.56272	1.21721	0.711734
Vimen/24	76.9333	53.37500	5.10335	21	0.000047	10.76016	10.09862	1.13531	0.909237
CD34/24	13.5333	7.62500	6.84596	21	0.000001	2.35635	0.74402	10.03011	0.005043
CD68/24	29.6000	22.25000	3.46462	21	0.002318	4.22239	5.89794	1.95112	0.272022

Tab. No. 2. Laser treated goupr after 24 vs. 72h

The result for hand instruments treated group versus laser treated group after 24h are shown on Tab. No.3.

	Mean	Std.Dv.	N	Diff.	Std.Dv.	t	df	p
Myelo/Cont/24.	58.1333	10.02758	15	-31.067	24.01626	-5.0100	14	0.000191
Myelo/Cont/72	89.2000	19.35828						
Vimen/Cont/24	53.2000	9.34421	15	-123.733	28.87774	-16.5947	14	0.000000
Vimen/Cont/72	176.9333	25.28259						
CD34Cont/24	8.7333	1.57963	15	-28.867	4.34029	-25.7587	14	0.000000
CD34/Cont/72	37.6000	4.98283						
CD68Cont/24	22.2667	5.21627	15	-44.200	11.34019	-15.0955	14	0.000000
CD68/Cont/72	66.4667	8.83877						

Tab. No. 3. Hand instrument treated group vs. Laser treated group after 24h.

The result shows significant differences ($p<0,001$) in all parameters. For CD34 and Vimentin was made a non-parametric analysis (Tab. No.4) and Mann-Whitney U test confirmed significant difference between the two groups for the parameter CD34 and Vimentin ($p <.05$).

	Rank Sum Contr.	Rank Sum Examin.	U	Z	p-level	Z adjusted	p-level	2*1sided exact p
CD34/24	240.0000	36.00000	0.00	3.872983	0.000108	3.902009	0.000095	0.000004
Vimen/72	226.5000	49.50000	13.50	3.001562	0.002686	3.006021	0.002647	0.001415

Tab. No. 4. Results for CD34 and Vimentin

Very similar results were obtained for the markers after 72h. Regarding the monitored parameters for Myeloperoxidase, Vimentin and CD68 average values are significantly higher for hand instruments treated group ($p<0,001$) while cell membrane positivity for the CD 34 antigen was detected as significantly higher for laser treated group ($p<0,001$) (Tab. No. 5.).

	Mean Conntr.	Mean Examin.	t-value	df	p	Std.Dev. Contr.	Std.Dev. Examin	F-ratio	p
Myelo/72	140.9333	93.8750	6.07525	21	0.000005	13.55658	23.90719	3.10997	0.067294
CD68/72	123.8667	65.7500	8.84964	21	0.000000	17.27453	8.84388	3.81528	0.081954
CD34/72	24.4667	35.2500	-4.52131	21	0.000187	5.96258	4.23421	1.98300	0.367037
Vimen/72	253.9333	178.8750	3.19090	21	0.004395	62.71417	28.18529	4.95092	0.040767

Tab. No. 5. Hand instrument treated group vs. Laser treated group after 72h.

4. DISCUSSION

Soft tissue curettage is technique established for removing the inner aspect of diseased gingival wall including the ulcerated and hyperplastic pocket epithelium, epithelial attachment and chronically inflamed connective tissue.(Novaes et al, 1969) Curettage essentially include conversion of the chronic inflammatory ulceration on the gingival wall of the pocket, into a surgical wound. Wound healing is process of tissue resolving and is includes continuous sequence of inflammation and repairing. During this process epithelial, endothelial, inflammatory cells, platelets and fibroblasts interact between them to restore normal function and structure of the tissue. Considering that, this study involved two methods of pocked debridement, and the expected outcome will be two types of wounding with different histological features and specifics of exposed underlying connective tissue: (1) "laser wounding" and (2) "curette wounding". The difference in wounding has influence on the obtained results about the early inflammatory response. The results of wound healing after 24/72 hours, according to our study, did showed significant difference for hand instruments versus laser treated group ($p<0,001$), for myeloperoxidase as a marker for neutrophil granulocytes, CD68 as monocytes/ macrophages marker and vimentin as a marker of mesenchymally-derived cells or cells undergoing an epithelial-to-mesenchymal transition. These findings indicate that there is an appointed inflammatory response following hand instruments treated group. Relatively low presence of marker proteins presented in our study, in early inflammatory response in laser treated gingival tissue can be result of the minimal invasive instrumentation of periodontal pocket without major trauma of the soft tissues. This findings are simmular with results published by Zaffe et al.(2004) Also, the results from previous investigations suggest that the extent of thermal alteration are different from tissue to tissue (Walsh et al, 1988) and the measured residual heat deposition values varied between 25-70% depending on the pulse duration. (Lukac et al, 2004) According to Lukac et al (2008) when using a thermal source, injury is thermal induced. Non-lethal tissue effects can be obtained at high temperatures when the heat source is maintained for a period of time less than that required to cause cell death. This generated non physiological level of heat is able to induce a heat shock response, which can be defined as the temporary changes in cellular activity.(Souil et al, 2001)

According to the results from this study, early phase of wound healing is followed by low energy ER: YAG laser assisted pocked debridement is characterized with relatively low postoperative (24 h) inflammatory markers response primarily due to the minimally invasive instrumentation of pocket without leading to major trauma of the soft tissues. Also, very narrow zone of thermal disruption, followed (in the first 72h) by increased values of CD34 transmembrane sialomucin proteins positive tissue cells are noted. As above mentioned CD34 is widely used as a marker of vascular endothelial cells, hematopoietic stem and progenitor cells. (Baumhueter et al, 1994; Young et al, 1995; Sato et al, 1999) Since the presence of CD34 positive cells in gingiva in stromal, paravascular location and basal layer of the gingival epithelium demonstrated in our study, the higher values for cell membrane positivity for CD34 protein for laser treated group after 72h could be interpreted as a positive signal activated during inflammation thus ensuring direction of appropriate cell types to area of inflammation in temporally correct manner. (Lavu et al, 2009) Recent scientific data show that healthy adult stem and progenitor cells improve the healing of wounds. Also, peripheral blood-derived $CD34^+$ cells, can accelerate the vascularization and healing of wounds. (Sivan-Loukianova et al, 2003) Detection of CD34 as a progenitor marker will allow further exploration of this particular subset of cells, which potentially possess a marked differentiation capacity.

5. CONCLUSION

The results from this study showed that low-level intensity of high power Er: YAG laser treatment may have a positive effect on entire healing, since the molecular cascade of becoming healthy, is similar for various tissue types in the human body. However, a number of further researches are needed before our understanding of sophisticated mechanisms of wound healing, after low –energy Er: YAG laser irradiation is complete.

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